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Identification and characterization of a new *Leishmania major* specific 3′nucleotidase/nuclease protein

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ABSTRACT

We report the characterization of a new *Leishmania major* gene, lmaj3′nt/nu, encoding a 382 amino acids protein, Lmaj3′NT/NU, that belongs to the 3′nucleotidase/nuclease family. Interestingly, sequence and phylogenetic analysis show that this protein is *Leishmania major* specific and thus constitutes a new 3′nucleotidase/nuclease subgroup. Lmaj3′NT/NU displays nuclease enzymatic activity and Western blot analysis shows that it is exclusively expressed in promastigotes. Immunofluorescence microscopy using a specific anti-Lmaj3′NT/NU shows that the protein has a plasma membrane localization. Surprisingly, contrary to the previously described *Leishmania mexicana* 3′NT/NU, lmaj3′nt/nu is not up-regulated when parasites are cultured under purine starvation conditions. Together, these findings suggest Lmaj3′NT/NU may constitute a new important compound of the *L. major* purine scavenging pathway and could be involved in sandfly parasite survival and colonization.

The Leishmaniasis are a heterogeneous group of diseases that affects millions of people in tropical and subtropical areas of the world (http://www.who.int/whr/en). Depending on the *Leishmania* parasite species and on the immunological response of the human host, disease ranges from asymptomatic infections to self-limiting cutaneous lesion(s) or fatal visceral forms. During their life cycle, parasites alternate between flagellated promastigotes in the midgut of the sandfly vector and amastigotes in the host macrophage [1]. The ability of *Leishmania* to survive and grow in different "hostile" environments such as the acidic phagolysosomal vesicles of mammalian macrophages and the midgut of sandflies indicates that it probably evolved adaptative strategies including extracellular nutrients acquisition, release of virulence factors, microbicidal resistance and evasion of host immune responses [2,3].

Leishmania, as other protozoa, are unable of *de novo* purine biosynthesis, hence for their survival in host environments, they developed a well organized pathway specialized in extracellular purine salvaging. Acquisition of host purines involves different membrane molecules including specialized purine nucleosides, nucleobase transporters and 3′nucleotidase/nuclease (3′NT/NU) proteins [4,5].

3′NT/NU is a family of proteins where the active site is exposed on the external face of the surface membrane. It displays two functional activities (3′nucleotidase and nuclease) and is well conserved in different *Leishmania* and kinetoplastid species [6–9]. Specific analogies in protein sequence and biochemical and kinetic properties were reported between 3′NT/NU and Class I single strand specific nucleases of plants and fungi. Several studies suggest that 3′NT/NU has a pivotal role in host purines scavenging by its capacity to generate free nucleosides via the hydrolysis of either exogenous 3′nucleotides or nucleic acids. Generated nucleosides are then incorporated into the parasite via specific nucleoside transporters.

Here, we report the characterization of a new *Leishmania major* gene, lmaj3′nt/nu, encoding a 382 amino acids protein, Lmaj3′NT/NU, that belongs to the 3′NT/NU family. Interestingly, sequence and phylogenetic analysis show that this protein is *L. major* specific and thus constitutes a new 3′NT/NU subgroup. Lmaj3′NT/NU is exclusively expressed in promastigotes, displays nuclease enzymatic activity and has plasma membrane localization. Surprisingly, contrary to the previously described *Leishmania mexicana* 3′NT/NU, lmaj3′nt/nu is not up-regulated when parasites are cultured under purine starvation conditions. Together, these findings suggest that Lmaj3′NT/NU may constitute a new compound of the *L. major* purine scavenging pathway that may play a key role in *Leishmania*–sandfly interaction.

Materials and methods

Parasites, culture conditions, genomic DNA preparations, and DNA sequencing. One *L. major* isolate (zymodeme MON-25; MHOM/TN/

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94/GLC94) taken from human ZCL lesion in El Guettar, Tunisia [10] and one *Leishmania infantum* (*Lcnj*) parasite strain, isolated from a dog with active canine VL, were used in this study. Promastigotes of *L. major* and *L. infantum* were cultured *in vitro* as described [11]. *Leishmania* stages (procyclics, stationary, metacyclics, and amastigotes) were obtained as described previously [11].

For purine depletion experiments, replete *L. major* promastigotes, supplied with a purine source, were grown in RPMI with 100 μM 3′AMP, 'adenosine-starved' cells were incubated in RPMI without FCS, and control parasites were grown in RPMI with FCS for various periods of time up to 3 days. In all experiments, cells were harvested by centrifugation as previously described [6,12].

Genomic DNA extraction and sequencing were performed as previously described [11].

Sequence and phylogenetic analysis. Searches for nucleotide and deduced amino acid sequences homologous to p6 transcript, potential p6 open reading frames (ORFs) and functional domains of P6 protein were performed as previously described [11]. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replications from the phylogeny package MEGA4 (www.megasoftware.net) [13].

Expression and purification of recombinant Lmaj3′NT/NU. In order to generate a specific antibody against Lmaj3′NT/NU, we expressed the carboxy-terminal part of the protein in *Escherichia coli*. The 3′ end of lmaj3′nt/nu DNA (709–1149 bp corresponding to aa 236–382) was PCR amplified using primers NucA (5′GCCCAT ATGGACCCGAAGCTGGTGAAG3′) and NucB (5′GCCGAATTCGCTT CCAGGTAGTCGACGAAC3′), then cloned into the pET-22b expression vector (Novagen, Fontenay-sous-Bois, France). The insoluble recombinant Lmaj3′NT/NUΔ235$(His)_6$ protein was then produced and purified as described previously [11]. Purity was demonstrated by SDS–polyacrylamide gel electrophoresis.

Preparation of antiserum and Western blotting. Antiserum against recombinant Lmaj3′NT/NUΔ235 was raised in immunized rabbits as previously described [11]. *L. major* or *L. infantum* proteins were separated by electrophoresis under native or denaturing conditions and transferred by electroblotting to nitrocellulose membranes (Amersham). Membranes were probed with the antiserum to Lmaj3′NT/NUΔ235 (diluted 1:3000). Bound anti-Lmaj3′NT/NUΔ235 antibodies were detected with a goat anti-rabbit secondary antibody coupled to peroxidase diluted 1:10,000 (Amersham) and revealed by peroxidase activity detection with the light-based ECL system following manufacturer's instructions (Amersham).

In situ staining for 3′nucleotidase and nuclease activities. 5×10^8 parasites from each stage (procyclic, stationary phase, metacyclic, and amastigotes) were lysed in 38 mM Tris, 100 mM glycine, pH 8.5, 1% SDS and 50 μg/ml leupeptin (Sigma) and separated by SDS–PAGE. Following renaturation, gels were stained *in situ* for 3′nucleotidase activity using 3′AMP as substrate [14,15]. Duplicate gels were stained *in situ* for nuclease activity using poly(A) as substrate [15,16].

Immunoprecipitation of the native Lmaj3′NT/NU. 2×10^9 *L. major* stationary phase promastigotes were lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet-P40 for 1 h at 4 °C. The soluble fraction was incubated with anti-rLmaj3′NT/NUΔ235 immune serum overnight at 4 °C then coupled to ProteinA–Sepharose (Amersham) for 2 h. Beads were then washed extensively [12], boiled in 1X gel sample buffer, separated by SDS–PAGE and stained *in situ* for enzyme activity as above.

Results

Identification of a new L. major 3′nucleotidase/nuclease-like

Using the mRNA differential display (DD), we previously identified several transcripts that are differentially expressed between high (HV) and low (LV) virulence *L. major* isolates [11,17,18]. For this study, we selected p6 transcript for a complete characterization since its ORF (p6 ORF) encodes an interesting 3′nucleotidase/nuclease-like protein. p6 was initially identified as overexpressed in *L. major* HV isolate in stationary phase promastigotes and its ORF corresponds to a large 1149 bp gene present as a single copy in the *L. major* genome (chromosome 31). It encodes a predicted 382 amino acids protein with a molecular weight of 41.7 kDa and an isoelectric point of 6.0. Cloning and sequencing of p6 ORF from the HV isolate reveals that the predicted P6 protein (GenBank Accession No. AY700936) is identical to the one from the Friedlin clone (LmjF31.2300). The deduced amino acid sequence of p6 ORF (Fig. 1A) exhibits significant homologies with proteins from the cell surface Class I nuclease family of several species (data not shown) and contains (i) a large domain (aa 29–300) that is highly conserved with S1/P1 nucleases, (ii) all five conserved amino acid signatures characteristic of the Class I nuclease family, (iii) a putative signal peptide (aa 1–23) and (iv) a carboxy-terminal trans-membrane region (aa 340–362).

P6 is a L. major specific protein

Blast sequence analysis reveals that *Leishmania*, *Trypanosoma*, and *Crithidia* include in their genomes several proteins belonging

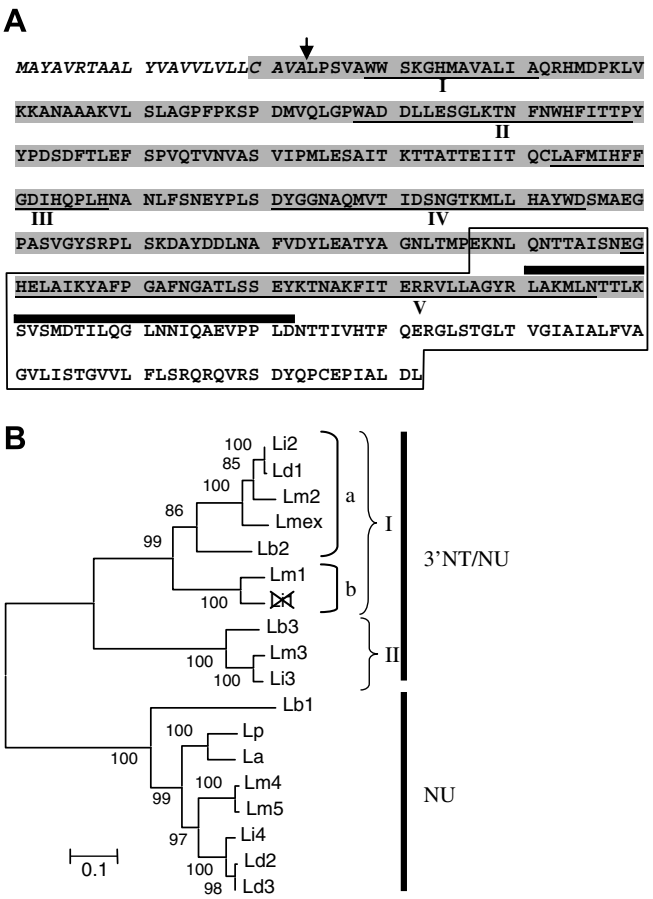

Fig. 1. Phylogenetic classification of *Leishmania* 3′NT/NU proteins. (A) Predicted protein sequence of P6 from *L. major* HV isolate. Italics: Signal peptide (aa 1–23). Arrowhead: Potential cleavage site (between aa 23 and 24). Grey shading: PI/SI nucleases homolog domain (aa 29–300). Underlined: The five signature sequences characteristic of Class I nucleases family (I to V). Black box: Putative trans-membrane anchor (aa 291–312). Boxed: Truncated protein produced in *E. coli* BL21 (aa 236–382). (B) *Leishmania* nuclease Class I phylogenetic tree constructed from 18 *Leishmania* sequences (Supplementary data S1) using the neighbor-joining method with 1000 bootstrap replications from the phylogeny package MEGA4. Numbers at individual nodes represent bootstrap support and the scale bar represents 0.1% divergence.

to the large nuclease Class I family displaying significant identities (28–89%) with P6 protein (data not shown). Thirty one of these proteins were selected on the basis of their homologies with P6, aligned and a phylogenetic tree was constructed using the neighbor-joining method. *Trypanosoma*, *Leishmania*, and *Crithidia* nuclease proteins organize into 4 distinct clusters (Supplementary data S1). Interestingly, *Leishmania* nucleases subdivide into 2 major clusters corresponding to two different nuclease groups: classical nuclease Class I and 3′NT/NU proteins that differ by the presence of domains located in the amino and carboxy-terminal part specific of each nuclease group (Class I and 3′NT/NU). The central part of these proteins exhibits motifs that are highly conserved (data not shown). Surprisingly, contrary to *Leishmania* and *Crithidia*, *Trypanosoma* do not seem to exhibit proteins of the 3′NT/NU family.

Further analysis of the *Leishmania* 3′NT/NU group allowed us to distinguish 2 subgroups (I and II) (Fig. 1B) differing by their sequences and the number of predicted carboxy-terminal membrane domains (I, $n = 1$ and II, $n = 2$). Moreover, subgroup I divides into two: a and b containing P6 protein and its *L. infantum* homolog (nucleotide identities 93%). Surprisingly, *L. infantum* p6 homolog found in databases contains an early STOP codon (29th codon) (Supplementary data S2). This result was experimentally confirmed by sequencing p6 from a Tunisian *L. infantum* strain. All these findings strongly suggest that P6 can be considered as a 3′nucleotidase/nuclease-like protein specific of *L. major* (Lmaj3′NT/NU).

Gene expression analysis and subcellular localization of the native Lmaj3′NT/NU

We characterized Lmaj3′NT/NU protein expression in different HV parasite stages using specific antibodies raised in rabbits immunized with the purified carboxy-terminal part of Lmaj3′NT/NU (deleted from the first aa 235; Lmaj3′NT/NUΔ235) produced in *E. coli*. We used a truncated protein since the whole Lmaj3′NT/NU protein is highly toxic for *E. coli*. We decided to analyze Lmaj3′NT/NU expression using non denaturing gel conditions in order to totally eliminate cross-reactions between the Lmaj3′NT/NUΔ235 antibody (anti-Lmaj3′NT/NUΔ235) and other *L. major* nuclease Class I proteins (data not shown).

Unlike pre-immune sera, anti-Lmaj3′NT/NUΔ235 strongly reacts with the recombinant truncated protein (data not shown). In *L. major* extracts, this serum also strongly recognizes a unique specific band of the expected size (42 kDa) (Fig. 2A). Moreover, bands of the same intensity are revealed in 10^7 procyclic and metacyclic *L. major* parasite extracts (Fig. 2A, lanes 1 and 2). However, this protein is absent in 10^7 amastigote lysates (Fig. 2A, lane 3). Interestingly, we definitely confirm that Lmaj3′NT/NU is *L. major* specific. Indeed, no band corresponding to an Lmaj3′NT/NU homolog is detected in *L. infantum* procyclic and metacyclic extracts (Fig. 2A, lanes 4 and 5).

Immunofluorescence microscopy of *Leishmania* parasites under permeabilized conditions using the anti-Lmaj3′NT/NUΔ235 shows a diffuse localization in the whole promastigote. In addition, we observe a large fluorescent spot closely adjacent and partly superimposed to the kinetoplast. This spot may correspond to flagellar pocket localization due to the presence of a signal peptide in Lmaj3′NT/NU. In non-permeabilized parasites, only a diffuse fluorescence is observed suggesting an Lmaj3′NT/NU plasma membrane localization (Supplementary data S3).

Nucleotidase and nuclease activities patterns in L. major and L. infantum parasites

In order to further analyze the polymorphism of 3′NT/NU proteins in *Leishmania*, we used *in situ* staining techniques to compare *L. major* and *L. infantum* nuclease and nucleotidase activities. A unique band with a similar size (42 kDa) corresponding to *L. major* and *L. infantum* 3′NT/NU proteins is detected for both activities (Fig. 2B, lanes 1, 2, 4, and 5). The absence of polymorphism between 3′ NT/NU activity patterns is probably due to the presence of 3 genes in *L major* and 2 in *L. infantum* with close molecular weights (40.9–41.7 kDa) (Supplementary data S4). A significant polymorphism is however observed for other *Leishmania* nuclease and/or nucleotidase proteins of 120 kDa and 29–31 kDa (Fig. 2B, lanes 1 and 2). These differences are related to the size, level of activity and/or number of proteins.

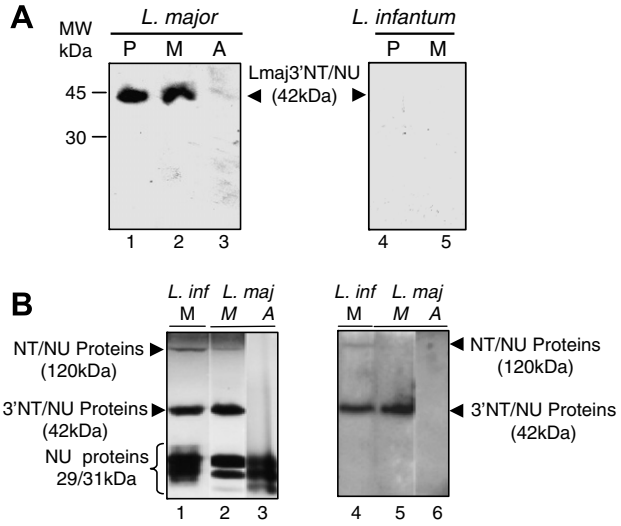

Fig. 2. Analysis of Lmaj3′NT/NU protein expression at different *Leishmania* developmental stages. (A) Lmaj3′NT/NU expression analysis using Western blot at different *Leishmania* developmental stages. 10^7 *L. major* and *L. infantum* procyclics (P) (lanes 1 and 4), metacyclics (M) (lanes 2 and 5) and 10^7 *L. major* amastigotes (A) (lane 3) were analyzed under non denaturing conditions. The blot was immunostained with the anti-Lmaj3′NT/NUΔ235. Arrowhead (◀): Native Lmaj3′NT/NU. (B) Analysis of *Leishmania* 3′NT/NU enzymatic activities: SDS–PAGE gels stained *in situ* for nuclease (lanes 1–3) and 3′nucleotidase (lanes 4–6) activities using 3′AMP and poly(A) as substrates, respectively. Enzymatic activities of 10^7 parasite lysates from *L. infantum* metacyclics (lanes 1 and 4), *L. major* metacyclics (lanes 2 and 5) and amastigotes (lanes 3 and 5) are analyzed in this figure. Arrowheads and bracket indicate parasite proteins that display nuclease and/or 3′nucleotidase activities.

To determine if a correlation exists between Lmaj3′NT/NU expression and *L. major* 3′NT/NU activity levels, we analyzed *L. major* 3′NT/NU activity patterns in metacyclic and amastigote parasites. Interestingly, contrary to metacyclic parasites where both nuclease and nucleotidase activities are present, no enzymatic activities are detected in amastigotes (Fig. 2B, lanes 3 and 6). These results indicate that Lmaj3′NT/NU expression levels correlate with 3′NT/NU activity levels and that these proteins are promastigote specific.

We then analyzed the potential nuclease and nucleotidase activities of Lmaj3′NT/NU. For this, we used a purified recombinant Lmaj3′NT/NU synthesized in the baculovirus system (Supplementary data S4) and the native protein purified by immunoprecipitation on *L. major* lysates, using the anti-Lmaj3′NT/NUΔ235. As shown in Fig. 3A, (lanes 2 and 3) only the native Lmaj3′NT/NU displays nuclease activity. No nucleotidase activity was detected for proteins tested (data not shown). We cannot exclude that the absence of such activity on immunoprecipitated Lmaj3′NT/NU could be due to the small quantities and the low sensitivity of the enzymatic assay. We were surprised that Lmaj3′NT/NU synthesized in the baculovirus system did not exhibit any enzymatic activity despite using high quantities of protein (data not shown). This can probably be explained by a difference in post-translational modifi-

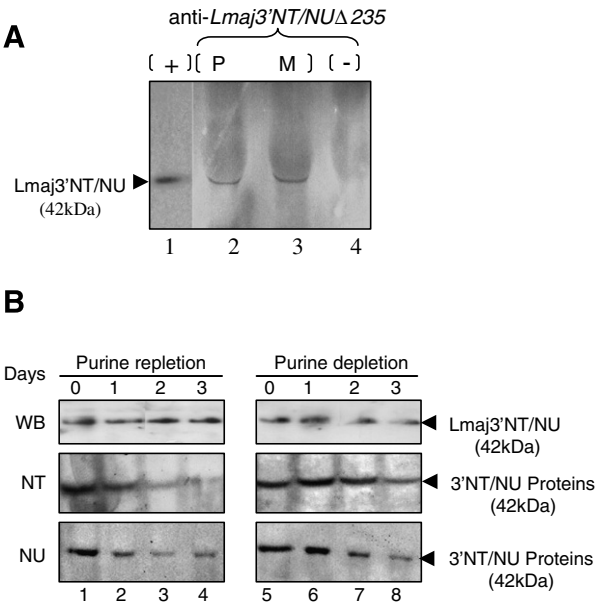

Fig. 3. Native Lmaj3′NT/NU nuclease activity and lmaj3′nt/nu gene expression under purine starvation culture conditions (A) nuclease activity displayed by the native Lmaj3′NT/NU immunoprecipitated with the anti-Lmaj3′NT/NUΔ235 from *L. major* procyclic and metacyclic promastigotes (lanes 2 and 3). Positive control: *L. major* metacyclic lysates (lane 1). Negative control: anti-Lmaj3′NT/NUΔ235 alone (lane 4). Arrowhead (◀): Immunoprecipitated 42 kDa Lmaj3′NT/NU. (B) Concomitant analysis of lmaj3′nt/nu expression and 3′nucleotidase and nuclease activities in *L. major* procyclic promastigotes cultured under purine starvation. *L. major* parasites are analyzed daily during three days (days 0–3) under purine repletion (lanes 1–4) or depletion conditions (lanes 5–8) by Western blot with the anti-Lmaj3′NT/NUΔ235 (WB) and *in situ* gel staining for 3′nucleotidase (NT) and nuclease (NU) activities. Arrowheads (◀): Lmaj3′NT/NU protein (WB) and *L. major* 3′NT/NU that display enzymatic activities (3′NT and NU).

cations of newly synthesized Lmaj3′NT/NU in *Leishmania* and in infected insect SF9 cells.

lmaj3′nt/nu gene expression in response to purine starvation

One interesting feature of trypanosomatidae 3′NT/NU genes is their up-regulation under purine starvation conditions [6,19]. In order to analyze this in *L. major*, we compared simultaneously the expression of lmaj3′nt/nu gene and the enzymatic activities of *L. major* 3′NT/NU in response to purine depletion. Exponential promastigotes were cultured during three days in RPMI without FCS (purine depletion) or without FCS plus 3′AMP (purine repletion). Surprisingly, under purine depletion conditions (Fig. 3B, WB, lanes 5–8), there is no significant up-regulation of lmaj3′nt/nu gene expression from day 1–3 compared to purine repletion conditions (Fig. 3B, WB, lanes 1–4) or to the positive control (parasites cultured in RPMI with FCS, data not shown). Moreover, from day 2, lmaj3′nt/nu is clearly less expressed under depletion (Fig. 3B, WB, lanes 7–8) than repletion (Fig. 3B, lanes 3–4) or control conditions (data not shown).

For *L. major* 3′NT and NU activities under starvation conditions, we observe the highest activity at day 1 followed by a progressive reduction from day 2. Interestingly, at days 1 and 2 these activities are higher than under purine repletion conditions (Fig. 3B, NU/NT, lanes 6–7; lanes 2–3) or in the positive control (data not shown). On the contrary, under repletion conditions, the enzymatic activities decrease significantly from day 1 and are weak at days 2–3. Interestingly, no clear correlation exists between lmaj3′nt/nu gene expression and *L. major* 3′ NT/NU enzymatic activities under purine depletion/repletion conditions. On the other hand, 3′NT and NU activity levels correlate perfectly under both conditions.

The discrepancy between lmaj3′nt/nu expression and *L. major* 3′NT/NU activities may be attributed to the presence of at least three 3′NT/NU genes in *L. major* genome and therefore one or more of them could be up-regulated.

Discussion

Leishmania parasites are purine auxotrophes. For their survival in hosts, they developed a highly specialized pathway involved in extracellular purine acquisition and recycling. 3′NT/NU proteins have been suggested to be an integral member of this pathway [7]. At present, only 2 proteins belonging to the 3′NT/NU subfamily were experimentally described in *Leishmania donovani* and *L. mexicana* [6,19]. However, their exact function in host purine acquisition still has to be demonstrated. Moreover, few data referring to the number, diversity, complexity, classification and functions of *Leishmania* 3′NT/NU proteins were published.

Here, we focused on the characterization of a new *L. major* specific protein, Lmaj3′NT/NU. Sequence analysis and phylogenetic studies allowed us to classify different 3′NT/NU and Class I nucleases from annoted trypanosomatids databases. We show that *Leishmania* and *Crithidia* 3′NT/NU constitute a homogeneous subfamily that differ from other trypanosomatids Class I nucleases by (i) the protein size (i.e. *Leishmania* Class I nucleases are aa 315–316 and 3′NT/NU up to aa 377), and the presence of (ii) at least one carboxy-terminal trans-membrane domain and (iii) specific regions in the amino and carboxy-terminal parts. Interestingly, we show that Lmaj3′NT/NU and its homolog in *L. infantum* (linf3′NT/NU) (89% of identities) form a new 3′NT/NU distinct subgroup. However, we experimentally demonstrate that, due to the presence of an early STOP codon, the whole Linf3′NT/NU is not produced. Lmaj3′NT/NU thus constitutes a unique member of this subgroup. Such gene polymorphism between two close *Leishmania* species (ORF genes display an average of 94% nucleotide identity) is very interesting in that it may partly contribute to better understand the evolution strategies adopted by different parasite species to govern specificity for their invertebrate hosts. As expected, we showed using immunoprecipitation experiments that the native Lmaj3′NT/NU displays nuclease enzymatic activity. However, despite the high relative conservation with previously characterized 3′NT/NU proteins (61–62% identities), no 3′nucleotidase enzymatic activity was detected. This can be due to the low amount of immunoprecipitated protein and the low sensitivity of the nucleotidase enzymatic activity assay. For this reason, an overexpression of lmaj3′nt/nu gene through *Leishmania* transfection experiments could be used to definitively demonstrate this activity as previously shown for other *Leishmania* 3′NT/NU [9].

Concomitant analysis of Lmaj3′NT/NU gene expression and *L. major* 3′nucleotidase/nuclease enzymatic activities in different parasite developmental stages reveal that Lmaj3′NT/NU expression and 3′NT/NU activities correlate perfectly and are exclusively restricted to promastigotes as previously described for *L. donovani* and *L. mexicana* [6,7,20]. This strongly suggests that *Leishmania* 3′NT/NU are essentially involved in insect vector colonization. Sopwith et al. [6] propose these proteins could maximize parasites capacity to salvage purines from the sandfly midgut where digestion of blood meals occurs.

In order to better understand the function of lmaj3′nt/nu gene, we analyzed in parallel its expression and *L. major* 3′NT/NU enzymatic activities under purine starvation culture conditions. Unexpectedly, no significant up-regulation of lmaj3′nt/nu gene expression was observed. In addition, lmaj3′nt/nu expression does not correlate perfectly with nuclease and nucleotidase activity levels for which a significant increase was detected under purine starvation compared to purine repletion conditions. lmaj3′nt/nu gene functions thus do not

seem to be strictly associated with purine starvation conditions and high *L. major* 3′NT/NU enzymatic activities obtained under these conditions are most probably due to an overexpression of the other *L. major* 3′nt/nu genes (in total, 3 annotated in *L. major* genome, including lmaj3′nt/nu). These results are different from those obtained by Sopwith et al. [6] who observe an up-regulation of *L. mexicana* 3′NT/NU at mRNA levels under purine starvation conditions. This could be explained by the fact that *L. major* and *L. mexicana* proteins belong to two different 3′NT/NU subgroups and their functions could be different during parasite life cycle.

In conclusion, this work allowed the partial characterization of a new specific *L. major* 3′NT/NU and may contribute to the identification of key molecules involved in host purine salvaging pathway and thus in better understanding parasite strategies adopted to survive in sandflies. Moreover, to our knowledge, this is the first time that protozoa 3′NT/NUs are classified and we show that Lmaj3′NT/NU is *L. major* specific. Thus, this work may also contribute to better understand the specificity of a parasite for its vector. However, only deletion of lmaj3′nt/nu followed by sandfly infections experiments will allow determining its precise role in purine salvaging and sandfly infection and specificity. Such informations on sandfly infection are essential to help fighting leishmaniasis.

Acknowledgments

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.099.

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